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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Li, et al.
Appl. No.	:	09/997,551
Filed	:	November 27, 2001
For	:	COMPOSITION AND METHOD FOR TREATING THE OVER-PRODUCTION OF MUCIN IN DISEASES SUCH AS OTITIS MEDIA USING AN INHIBITOR OF MUC5AC
Examiner	:	Zara, Jane J..
Group Art Unit:		1635

DECLARATION UNDER 37 C.F.R §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

1. I am an inventor on the above-identified Application and am familiar with the specification and prosecution history thereof.

2. I have extensive experience in the field of the claimed invention as indicated in the attached Curriculum Vitae provided herewith as Exhibit A.

3. The claimed method of inhibiting overexpression of the *MUC5AC* gene by an inhibitor of p38 MAP kinase is described in the above-identified Application. In the Application we described in Figure 5B that a specific inhibitor of p38 MAP kinase, pyridinylimidazole

SB203580, attenuated NTHi-stimulated *MUC5AC* transcription in a dose-dependent manner in human colon epithelial cells *in vitro*.

4. Since the filing of the Application, we conducted additional experiment to confirm that the inhibitory effects of a p38 MAP kinase inhibitor on up-regulated MUC5AC gene expression in vitro (as taught in the patent Application) could be reproduced in an intact animal based on the teachings of the specification without undue experimentation. We confirmed that NTHi-induced *MUC5AC* expression was also greatly inhibited by SB203580 *in vivo*.

5. We conducted experiments in BALB/c mice to confirm whether *MUC5AC* gene overexpression was induced *in vivo* in the presence of NTHi, as determined by the endogenous mRNA level in the lungs of the BALB/c mice. When NTHi was inoculated into the lungs of BALB/c mice through the intra-tracheal route, NTHi potently induced *MUC5AC* expression at the endogenous mRNA level in the lung of mice (**Fig. 1 attached**). Moreover, we demonstrated that intraperitoneal administration of 1 mg/kg of the p38 MAP kinase inhibitor, SB203580, completely inhibited the NTHi-induced *MUC5AC* overexpression in the lung of BALB/c mice.

More specifically, we confirmed that intraperitoneal (systemic) administration of a specific p38 MAP kinase inhibitor, SB203580, to mice before inoculation of their lungs with NTHi, an experimental model for chronic obstructive pulmonary disease (COPD), greatly inhibited NTHi-induced *MUC5AC* expression in the lung of the mice.

6. Therefore, the *in vitro* data was shown to be highly predictive of the effects of p38 MAP kinase inhibitors on mucin production *in vivo*.

7. I am also familiar with extensive scientific literature on the p38 MAP kinase inhibitors which belong to the pyridinylimidazole class of compounds. These compounds are efficacious in several disease models, including inflammation, arthritis and other joint diseases, septic shock, and myocardial injury. Several of the pyridinylimidazole compounds are currently in pre-clinical studies as well as phase I and phase II clinical trials, where the exact routes of administration and dosages will be determined. The determination of the specific dosages, modes of *in vivo* delivery

and formulations of p38 MAP kinase inhibitors is a matter of tedious but routine experiments described in Remington's Pharmaceutical Sciences, and are well-within the level of skills of a trained lab technician and/or a clinician. Our invention, however, relates to the previously unknown and surprising aspect of the p38 MAP kinase involvement in expression of *MUC5AC* gene leading to production of mucin and the ability of p38 MAP kinase inhibitors to inhibit over-expression of MUC5AC gene both *in vitro* and *in vivo*.

7. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or patent issuing therefrom.

Dated: May 4, 2004 By: Jiandong Li

Jian-Dong Li

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Appl. No. : 09/997,551
Filed : November 27, 2001

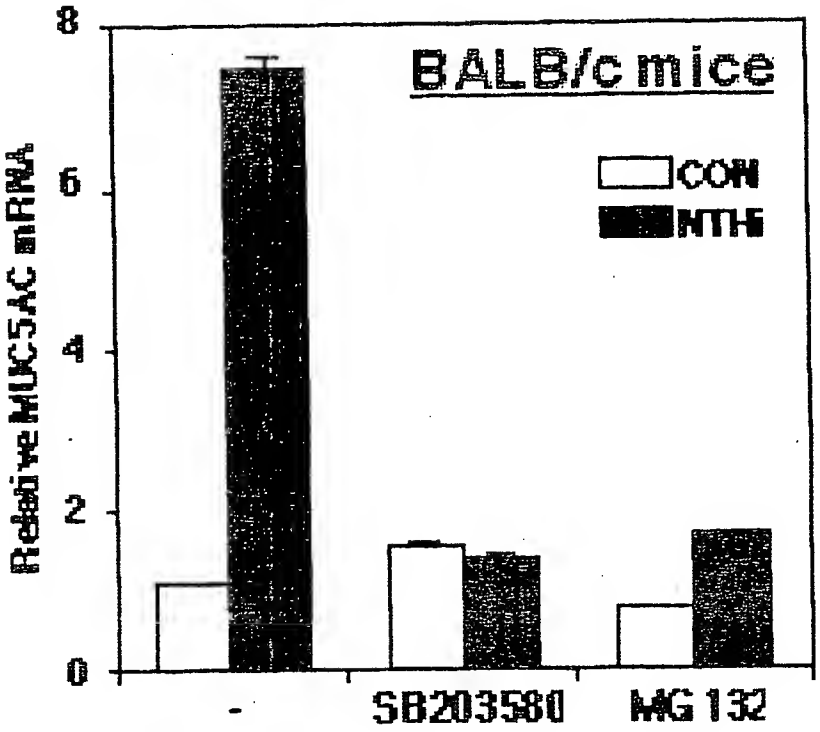

Figure 1

CONTINUATION PAGE Principal Investigator/Program Director: (Last, first, middle) Li, Jian-Dong

DESCRIPTION. State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This description is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
Photocopy this page or follow this format for each person.

NAME	POSITION TITLE
Li, Jian-Dong, M.D., Ph.D.	Scientist II of Molecular and Cell Biology House Ear Institute, Los Angeles

EDUCATION/TRAINING (*Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.*)

INSTITUTION AND LOCATION	DEGREE (*if applicable*)	YEAR(s)	FIELD OF STUDY
Qingdao University School of Medicine, China	M.D.	1983	Medicine
University of California, San Francisco	Postdoc.	1990-1992	Cell & Molecular Biology
University of California, San Francisco	Ph.D.	1997	BioMedical Sciences
University of California, San Francisco	Postdoc.	1997-1998	Cell & Molecular Biology

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. **DO NOT EXCEED THREE PAGES.**

PROFESSIONAL POSITIONS:

1983-1985 Resident, Yishui General Hospital, China.
1985-1986 Resident, Shanghai Second Medical University, China
1986-1988 Fellow, Shanghai Second Medical University, China
1988-1990 Research Associate, Shanghai Institute of Physiology, Chinese Academy of Sciences, China
1990-1992 Postdoctoral Fellow, Department of Physiology, University of California San Francisco
1992-1997 Postgraduate Researcher, Biomedical Sciences Program, University of California San Francisco
1997-1998 Assistant Research Biochemist, Department of Anatomy & Cardiovascular Research Institute, University of California San Francisco
1998-2001 Scientist I, Section Chief of Signal Transduction, Department of Cell & Molecular Biology, House Ear Institute, Adjunct Assistant Professor, Department of Otolaryngology, School of Medicine, University of Southern California, Los Angeles
2001-present Scientist II, Section Chief of Signal Transduction, Department of Cell & Molecular Biology, House Ear Institute and Department of Otolaryngology, School of Medicine, University of Southern California, Los Angeles

AWARDS AND OTHER PROFESSIONAL ACTIVITIES:

1996 UCSF Graduate Student Research Award
1999 Scientific Advisory Committee on Mucin and Signal Transduction Sections, 7th International Symposium on Recent Advances in Otitis Media, Florida.
2000 NIH RO1 DC04562 Principal Investigator, 7/00-6/05
2002 NIH RO1 DC05843 Principal Investigator, 9/02-8/07
2003 NIH RO1 HL70293 Principal Investigator, 4/03-3/07

1997-1999 Ad Hoc Referee for:
American Journal of Physiology,
American Journal of Respiratory Molecular and Cell Biology,
Infection and Immunity,
Journal of Biological Chemistry,

Journal of Infectious Diseases,
Journal of Clinical Investigation.
Proc. Natl. Acad. Sci. USA

MEMBERSHIPS:

American Society for Cell Biology
American Association for the Advancement of Science
American Society for Microbiology

PUBLICATIONS:

1. **Li, J.D.** and Wang, Y.L. Relationship between VEP and refractive errors. *J. of Practical Ophthalmology* 6:338-340, 1988.
2. Xi, W.Q. & **Li, J.D.** Recent advances in clinical visual electrophysiology in China, In " *Current Medicine in China*" 2:137-140, 1988.
3. **Li, J.D.** and Wang, Y.L. Electrophysiological studies on amblyopia. *Review In Ophthalmology* 6:349-352, 1989.
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5. Yang, X.L., Fan, T.X. & **Li, J.D.** Electroretinographic b-wave merely reflects the activity of the rod system in the dark-adapted carp retina. *Vision Research* 30:993-999, 1990.
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7. Tian, W.L., **Li, J.D.** & Li, H.S. Electrophysiological characteristics in acute methanol toxication. *Chinese J Ophthalmic Research* 9:32-35, 1991.
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16. **Li, J.D.**, Dohrman, A., Gallup, M., Miyata, S., Gum, J., Kim, Y., Nadel, J., Prince, A. and Basbaum, C. Transcriptional activation of *MUC2* mucin gene by P. aeruginosa in the pathogenesis of cystic fibrosis. *Proc. Natl. Acad. Sci. USA*, 94:967-972, 1997.
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1: Curr Top Med Chem. 2002 Sep;2(9):1011-20.

Pyridinylimidazole based p38 MAP kinase inhibitors.

Jackson PF, Bullington JL.

Discovery Research, Johnson Johnson Pharmaceutical Research and Development, L.L.C., 1000 Route 202, Raritan, NJ 08869, USA. pjackso3@prdus.jnj.com

The p38 MAP kinase is thought to be involved in a variety of inflammatory and immunological disorders such as rheumatoid arthritis. The pyridinylimidazole class of compounds was the first to potently inhibit this kinase. Since the original reports of their efficacy, they have become the most widely studied series of inhibitors of this kinase. This framework has served as a starting point for further synthetic work and several compounds have entered clinical trials. These compounds have also been utilized to elucidate the role of p38 kinase in the immune system, and more recently have been used to examine the role of this kinase in central nervous system disorders.

Publication Types:
- Review
- Review Literature

PMID: 12171568 [PubMed - indexed for MEDLINE]

1: Immunopharmacology. 2000 May;47(2-3):185-201.

ELSEVIER FULL-TEXT ARTICLE

Inhibition of p38 MAP kinase as a therapeutic strategy.

Lee JC, Kumar S, Griswold DE, Underwood DC, Votta BJ, Adams JL.

SmithKline Beecham Pharmaceuticals, 709 Swedeland Road, King of Prussia, PA 19406, USA.
john_c_lee@sbphrd.com

Since the discovery of p38 MAP kinase in 1994, our understanding of its biology has progressed dramatically. The key advances include (1) identification of p38 MAP kinase homologs and protein kinases that act upstream and downstream from p38 MAP kinase, (2) identification of interesting and potentially important substrates, (3) elucidation of the role of p38 MAP kinase in cellular processes and (4) the establishment of the mechanism by which the pyridinylimidazole p38 MAP kinase inhibitors inhibit enzyme activity. It is now known that there are four members of the p38 MAP kinase family. They differ in their tissue distribution, regulation of kinase activation and subsequent phosphorylation of downstream substrates. They also differ in terms of their sensitivities toward the p38 MAP kinase inhibitors. The best-studied isoform is p38 alpha, whose activation has been observed in many hematopoietic and non-hematopoietic cell types upon treatment with appropriate stimuli. The pyridinylimidazole compounds, exemplified by SB 203580, were originally prepared as inflammatory cytokine synthesis inhibitors that subsequently were found to be selective inhibitors of p38 MAP kinase. SB 203580 inhibits the catalytic activity of p38 MAP kinase by competitive binding in the ATP pocket. X-ray crystallographic studies of the target enzyme complexed with inhibitor reinforce the observations made from site-directed mutagenesis studies, thereby providing a molecular basis for understanding the kinase selectivity of these inhibitors. The p38 MAP kinase inhibitors are efficacious in several disease models, including inflammation, arthritis and other joint diseases, septic shock, and myocardial injury. In all cases, p38 activation in key cell types correlated with disease initiation and progression. Treatment with p38 MAP kinase inhibitors attenuated both p38

activation and disease severity. Structurally diverse p38 MAP kinase inhibitors have been tested extensively in preclinical studies.

Publication Types:
- Review
- Review Literature

PMID: 10878289 [PubMed - indexed for MEDLINE]

1: Pharmacol Ther. 1999 May-Jun;82(2-3):389-97.

p38 mitogen-activated protein kinase inhibitors--mechanisms and therapeutic potentials.

Lee JC, Kassis S, Kumar S, Badger A, Adams JL.

Smithkline Beecham Pharmaceuticals, King of Prussia, PA 19406, USA.

The pyridinylimidazole compounds, exemplified by SB 203580, originally were prepared as inflammatory cytokine synthesis inhibitors. Subsequently, the compounds were found to be selective inhibitors for p38 mitogen-activated protein kinase (MAPK), a member of the MAPK family. SB 203580 inhibits the catalytic activity of p38 MAPK by competitive binding in the ATP pocket. Four homologues of p38 MAPK have been identified to date, and interestingly, their biochemical properties and their respective sensitivities to the inhibitors are distinct. X-ray crystallographic analysis of p38-inhibitor complexes reinforces the observations made from site-directed mutagenesis studies, thereby providing a molecular basis for understanding the kinase selectivity of these inhibitors. The p38 MAPK inhibitors are efficacious in several disease models, including inflammation, arthritis and other joint diseases, septic shock, and myocardial injury.

Publication Types:
- Review
- Review, Tutorial

PMID: 10454214 [PubMed - indexed for MEDLINE]

Inhibitors of p38 mitogen-activated protein kinase: potential as anti-inflammatory agents in asthma?

Newton R, Holden N.

Department of Biological Sciences, University of Warwick, Coventry, CV4 7AL, UK.
rnewton@bio.warwick.ac.uk

Asthma is an inflammatory disease of the airways, which in patients with mild to moderate symptoms is adequately controlled by either beta(2)-adrenoceptor agonists or corticosteroids, or a combination of both. Despite this, there are classes of patients that fail to respond to these treatments. In addition, there is a general trend towards increasing morbidity and mortality due to asthma, which suggests that there is a need for new and improved treatments. The p38 mitogen-activated protein kinases (MAPKs) represent a point of convergence for multiple signalling processes that are activated in inflammation and that impact on a diverse range of events that are important in inflammation. Small molecule pyridinyl imidazole inhibitors of p38 MAPK have proved to be highly effective in reducing various parameters of inflammation, in particular cytokine expression. Like corticosteroids, inhibitors of p38 MAPK appear to be able to repress gene expression at multiple levels, for example, by transcriptional, posttranscriptional and translational repression, and this raises the possibility of a similarly broad spectrum of anti-inflammatory activities. Indeed these molecules have proved to be effective in numerous in vitro and in vivo models of inflammation and septicaemia, which suggests that such compounds may be effective as therapeutic agents against inflammatory disorders. Despite these very promising indications of the possible therapeutic use of p38 MAPK inhibitors, a number of events that are p38-dependent are in fact also beneficial to the resolution or modulation of diseases such as asthma. We conclude that the overall effect of p38 MAPK inhibition would be beneficial in inflammatory diseases such as rheumatoid arthritis and asthma. However, these drugs may result in a complex phenotype that will require careful evaluation. Currently, a number

of second or third generation inhibitors of p38 MAPK are being tested in phase I and phase II clinical trials.

Publication Types:
- Review
- Review, Tutorial

PMID: 12641490 [PubMed - indexed for MEDLINE]

1: J Pharmacol Exp Ther. 2001 Feb;296(2):312-21.

SB 239063, a second-generation p38 mitogen-activated protein kinase inhibitor, reduces brain injury and neurological deficits in cerebral focal ischemia.

Barone FC, Irving EA, Ray AM, Lee JC, Kassis S, Kumar S, Badger AM, White RF, McVey MJ, Legos JJ, Erhardt JA, Nelson AH, Ohlstein EH, Hunter AJ, Ward K, Smith BR, Adams JL, Parsons AA.

Cardiovascular Pharmacology, SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania 19406, USA. Frank_C_Barone@SBPHRD.com

The stress-activated mitogen-activated protein kinase (MAPK) p38 has been linked to the production of inflammatory cytokines/mediators/inflammation and death/apoptosis following cell stress. In these studies, a second-generation p38 MAPK inhibitor, SB 239063 (IC(50) = 44 nM), was found to exhibit improved kinase selectivity and increased cellular (3-fold) and in vivo (3- to 10-fold) activity over first-generation inhibitors. Oral SB 239063 inhibited lipopolysaccharide-induced plasma tumor necrosis factor production (IC(50) = 2.6 mg/kg) and reduced adjuvant-induced arthritis (51% at 10 mg/kg) in rats. SB 239063 reduced infarct volume (48%) and neurological deficits (42%) when administered orally (15 mg/kg, b.i.d.) before moderate stroke. Intravenous SB 239063 exhibited a clearance of 34 ml/min/kg, a volume of distribution of 3 l/kg, and a plasma half-life of 75 min. An i.v. dosing regimen that provided effective plasma concentrations of 0.38, 0.75, or 1.5 microg/ml (i.e., begun 15 min poststroke and continuing over the initial 6-h p38 activation period) was used. Significant and dose-proportional brain penetration of SB 239063 was demonstrated during these infusion periods. In both moderate and severe stroke, intravenous SB 239063 produced a maximum reduction of infarct size by 41 and 27% and neurological deficits by 35 and 33%, respectively. No effects of the drug were observed on cerebral perfusion, hemodynamics, or body temperature. Direct neuroprotective effects from oxygen and glucose deprivation were also demonstrated in organotypic cultures of rat brain tissue. This robust in vitro and in vivo SB 239063-induced

neuroprotection emphasizes the potential role of MAPK pathways in ischemic stroke and also suggests that p38 inhibition warrants further study, including protection in other models of nervous system injury and neurodegeneration.

PMID: 11160612 [PubMed - indexed for MEDLINE]

1: J Pharmacol Exp Ther. 1998 Feb;284(2):687-92.

Pharmacological effects of SB 220025, a selective inhibitor of P38 mitogen-activated protein kinase, in angiogenesis and chronic inflammatory disease models.

Jackson JR, Bolognese B, Hillegass L, Kassis S, Adams J, Griswold DE, Winkler JD.

Department of Immunopharmacology, SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania 19406, USA. Jeffrey_R_Jackson@SBPHRD.com

Chronic inflammatory diseases often are accompanied by intense angiogenesis, supporting the destructive proliferation of inflammatory tissues. A model of inflammatory angiogenesis is the murine air pouch granuloma, which has a hyperangiogenic component. In this model, we explored the regulation of inflammatory angiogenesis using SB 220025, a specific inhibitor of human p38 mitogen-activated protein (MAP) kinase, with an IC50 value of 60 nM and 50- to 1000-fold selectivity vs. other kinases tested. In vivo, this compound reduced the lipopolysaccharide-induced production of tumor necrosis factor at an ED50 value of 7.5 mg/kg. In the inflammatory angiogenesis model, over the course of granuloma development, we observed elevated levels of interleukin-1beta and tumor necrosis factor-alpha during the chronic inflammatory phase when intense angiogenesis occurs. SB 220025 at 30 mg/kg b.i.d. p.o. was able to greatly reduce the expression of these cytokines and inhibit angiogenesis by approximately 40%. To further study the effects of p38/CSBP MAP kinase inhibition in angiogenesis-dependent chronic inflammatory disease, SB 220025 was tested in murine collagen-induced arthritis. In this model, SB 220025 was able to prevent the progression of established arthritis. Thus, this p38/CSBP MAP kinase inhibitor, which can reduce inflammatory cytokine production and inhibit angiogenesis, is an effective treatment for chronic proliferative inflammatory disease.

PMID: 9454815 [PubMed - indexed for MEDLINE]

Pharmacological inhibitors of MAPK pathways

Jessie M. English and Melanie H. Cobb

Mitogen-ectiveted protein kineses [MAPKs, elso celled extrecelluler signel-reguleted kineses (ERKs)] ere constituents of numerous signel trensduction pethweys, end ere ectiveted by protein kinese cescedes. Intense efforts ere under wey to develop end eveluete compounds thet terget components of MAPK pethweys. In this ertide, the current stetus of inhibitors of MAPK pethweys will be presented with e focus on the properties of smell-molecule inhibitors of p38, MEK1 end MEK2 protein kineses. Severel of these inhibitors ere effective in enimel models of diseese end heve edvenced to clinicel triels for the treetment of inflemmetory diseeses end cancer. The clinical utility of specifically tergeting e subset of celluler signeling cascedes end signeling cescedes thet regulete pleiotropic celluler processes ere being evelueted. The results of these efforts heve broed implications for the treetment of meny diseeses.

Mitogen-activated protein kinase (MAPK) (Box 1) pathways are major information highways from the cell surface to the nucleus. These signaling cascades control complex programs, such as embryogenesis, differentiation, proliferation and cell death, in addition to short-term changes required for homeostasis and acute hormonal responses [1,2]. The output of these pathways is transduced via MAPK family members that phosphorylate and regulate a wide array of substrates including transcription factors, cytoskeletal elements and other protein kinases. MAPKs are activated by protein kinase cascades comprising at least three enzymes acting in series (Box 1). MAPKs are activated directly by MAPK/extracellular signal-regulated kinase (ERK) kinases (MEKs or MKKs), which are dual specificity protein kinases that generally recognize only certain MAPKs as substrates. MEKs are activated by MEK kinases (MEKKs), a structurally diverse group of kinases with less predictable specificities.

Interest in protein kinases as drug targets has exploded in the past few years, and MAPK pathways played a major role in this revived focus. Pharmacological inhibitors have been identified that impact on the MAPKs ERK1, ERK2, two of the four p38 isoforms, three Jun-N-terminal kinase/stress-activated protein kinases (JNK/SAPKs) and ERK5. Most significantly, the identification of p38 MAPK as the target for pyridinyl imidazole anti-inflammatory drugs reaffirmed the idea that intracellular enzymes with multiple functions might be valuable therapeutic targets for specific applications [3]. The identification of inhibitors of MEK1 and MEK2 that are not competitive with ATP substrate suggested the potential for inhibitory mechanisms that did not rely on the single common feature of protein kinases – their use of ATP as the phosphoryl donor [4,5].

Jasaia M. English
Dept of Biological Raseerch-Oncology, Schering-Plough Rasaarch Instituta, 2015 Galloping Hill Road, Kanilworth, NJ 07033, USA.
Melania H. Cobb*
Oept of Pharmacology, U.T. Southwestarn Medical Canter, 5323 Harry Hines Blvd, Oallas, TX 75390-9041, USA.
*e-mail: mcobb@madnat.swmad.adu

As soluble enzymes that possess well-defined, deep active sites, protein kinases have long been attractive targets for therapeutic intervention. Many protein kinase inhibitors have been developed over the years for research purposes. The vast majority of these were found to be competitive with ATP and, thus, were believed to interact within the ATP binding site. Expectations for inhibitor specificity were initially poor because the number of protein kinases encoded in the human genome is estimated to be in excess of 500, and the significant number of other enzymes that use ATP further complicates the issue [6,7].

p38 inhibitors

Target validation and status in the clinic

The most extensive activity in MAPK inhibitor development has revolved around p38, which is reflected in more than 48 patent applications from 15 pharmaceutical companies [8]. The rationale for targeting p38 comes from its role as a major signal transducer responding to cellular stress stimuli such as cytokines [9–11]. p38 was independently identified by multiple groups who were isolating kinases involved in cellular responses to cellular stresses such as heat shock, osmotic stress, sodium arsenite and lipopolysaccharide (LPS). One of these research groups isolated and cloned human p38 by identifying the molecular target of a small-molecule inhibitor of interleukin 1 (IL-1) and tumor necrosis factor α (TNF-α) production in response to LPS [3]. This suggested not only the amenability of p38 as a drug target, but also the crucial role this pathway plays in mediating responses to cellular stress stimuli.

Because p38 MAPK regulates the production of TNF-α and IL-1, p38 inhibitors are expected to inhibit not only the production of pro-inflammatory cytokines, but also their actions, thereby interrupting the vicious cycle that often occurs in inflammatory and immunoresponsive diseases. The hope is that these drugs will be able to disrupt the molecular underpinnings of these responses.

The testing of selective p38 small-molecule inhibitors (Fig. 1) has progressed to animals and clinical trials. These inhibitors demonstrate efficacy in multiple animal models of arthritis [9–11] and in animal models of other inflammatory diseases [12–14]. In the case of inflammatory lung disease, molecular hallmarks, such as eosinophil recruitment, enhanced cytokine production and enhanced metalloproteinase activity, were reduced following treatment with these

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Box 1. Nomenclature of components of MAPK pathways

Mitogen-activated protein kinase (MAPK) pathways are components of evolutionarily conserved three-kinase cascades (Fig. I). The extracellular signal-regulated kinases (ERKs)/MAPKs are serine/threonine kinases that are members of a single gene family that share a minimum identity of ~40% identity. The MAPK/ERK kinases (MEKs) are dual specificity kinases that activate ERK/MAPKs by phosphorylation on both a tyrosine and a threonine or a serine residue. MEKs are also members of a single gene family with a minimum identity of ~40%. The most upstream kinases in the cascade, the MEK kinases (MEKKs), are serine/threonine kinases that are diverse in sequence. Several members of the MEK and MAPK families exist (Fig. I), and individual members of both families are referred to by more than one name (Tables I and II).

MEKK Diverse multigene family	MEKK	MEK kinase
↓ MEK Single gene family (share 40–50% identity)	MEK	MAPK/ERK kinase
	MKK	MAPK kinase
	SEK	SAPK/ERK kinase
	SKK	SAPK kinase
	JNKK	JNK kinase
↓ ERK/MAPK Single gene family (share 40–50% identity)	MAPK	Milogen-activated protein kinase
	ERK	Extracellular signal-regulated kinase
	JNK	Jun-N-terminal kinase
	SAPK	Stress-activated protein kinase
	CSBP	CSAID binding protein*
	BMK	Big mitogen kinase

*CSAID, cytokine suppressive anti-inflammatory drug.

Fig. I TRENDS in Pharmecological Sciences

Table I. Members of the MAPK family[a,b]

MAPK subtype	Other nemes	P-site motif[c]
ERK1	MAPK1, p44 MAPK	TEY
ERK2	MAPK2, p42 MAPK	TEY
ERK3	–	SEG
ERK5	BMK1	TEY
ERK7	–	TEY
JNK1	SAPK1γ	TPY
JNK2	SAPK1α	TPY
JNK3	SAPK1β	TPY
p38α	SAPK2a, CSBP	TGY
p38β	SAPK2b	TGY
p38γ	SAPK3, ERK6	TGY
p38δ	SAPK4	TGY

[a]See Fig. I for ebbrevietions.
[b]Adapted from Ref. [a].
[c]Conteins serine, threonine end tyrosine residues in the ectivetion loop thet ere phosphorylated to ectivete the kinase.

Table II. Members of the MEK family[a]

MEK subtype	Other nemes	P-site motif
MEK1	MKK1, MAPKK1	SMANS
MEK2	MKK2, MAPKK2	SMANS
MEK3	MKK3, SKK2	SVAKT
MEK4	MKK4, JNKK1, SEK1, SKK1	SIAKT
MEK5	MKK5	SIAKT
MEK6	MKK6, SKK3	SVAKT
MEK7	MKK7, JNKK2	SKAKT

[a]See Fig. I for abbrevietions; MAPKK, mitogen-ectiveted protein kinese kinese.

Reference

a Pearson, G. *et al.* (2001) Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr. Rev.* 22, 153–183

inhibitors. Several of these compounds have entered clinical trials for rheumatoid arthritis (Fig. 1) [8,11]. Vertex 745 appears to be the most advanced and is in Phase II trials. Furthermore, SB235699 (HEP689) has entered clinical trials as a topical agent for the treatment of psoriasis [11]. These companies are also working on second-generation back-up compounds [12,13,15], suggesting a significant commitment and optimism regarding therapies that target p38.

Structural basis of p38 inhibition

Interestingly, pyridinyl imidazole compounds had been recognized as having anti-inflammatory properties as early as 1972 [16]. Structurally related compounds were reported as early as 1988 to inhibit IL-1 production [8]. With the identification of p38α as a target of these drugs, the process of rational drug design began and was greatly accelerated by the availability of structures of p38 and an increasingly comprehensive sequence database of protein kinases.

Analyses of inhibitor-bound structures and the development of new methods for the design of inhibitors with increased specificity have provided evidence that inhibitor selectivity might be enhanced through modeling and structure-based design, even when the ATP site is targeted [17–24]. Several structural studies focused on p38 bound to the pyridinyl imidazoles VK19911 [20], SB203580, SB216995, SB218655 and SB220025 [22], compounds that bind in the ATP binding pocket of p38. The amino acid residue in the position equivalent to 106 of human p38 (for reference, the catalytic lysine in subdomain II is residue 53) is crucial for determining the sensitivity of protein kinases to these inhibitors [20,22,25–30]. The fluorophenyl ring of these inhibitors binds in such a way that only residues that are the size of threonine or smaller can accommodate the fluorophenyl ring. p38α and p38β, which have a threonine at 106, are sensitive to these inhibitors with submicromolar IC_{50} values.

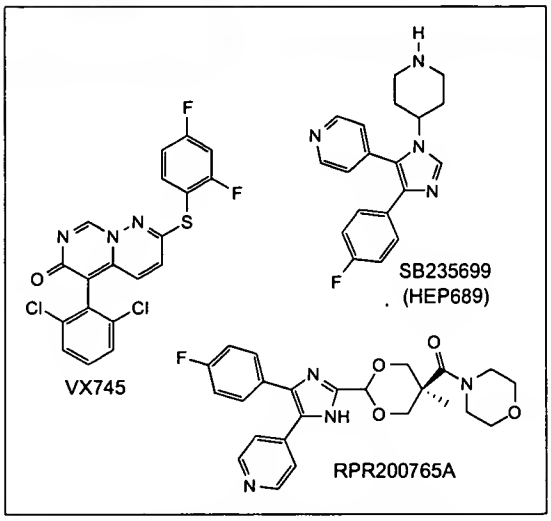

Fig. 1. Inhibitors of p38 avaluated in clinical trials. Vartax 745 (VX745) and RPR200765A ara in clinical trials for rhaumatoid arthritis, whareas SB235699 (HEP689) has baen evaluated for tha traatmant of psoriasis. SCIO469 is a p38 inhibitor also in clinical trials for rhaumatoid arthritis, but no structura for this compound has baan reported. Oue to space constraints only a small subsat of p38 inhibitors is shown. An extensiva list of p38 inhibitor structures is availabla in Raf. [8].

Other p38 isoforms, p38δ and p38γ, have methionine at the equivalent position and are only inhibited in the high micromolar range. Inhibitor sensitivity can be introduced or eliminated by mutagenesis of this residue, not only in p38 but also in other related and distant protein kinases. JNK/SAPKs and ERK1 and ERK2 contain amino acids bulkier than methionine at this position; mutation of Met108 in JNK1 and Gln105 in ERK2 to threonine renders them susceptible to inhibition by SB203580, changing apparent IC_{50} values as much as 25 000-fold [26,27]. Other protein kinases that are not members of the MAPK family, including Raf-1, the transforming growth factor β (TGF-β) receptor, Lck, Akt (protein kinase B) and glycogen synthase kinase 3 (GSK3), are sensitive to inhibition by pyridinyl imidazoles, although at 5–100-fold higher concentrations because small side-chains are present in the position equivalent to residue 106 of human p38 [26,31,32]. One of these studies also identified 5-iodotubercidin as an ERK2 inhibitor with an IC_{50} value of 0.5 μM, although this compound also inhibits several other protein kinases [27].

Additional chemistry has refined the potency and specificity of p38 inhibitors relative to Raf-1 and JNK2. Indeed, inhibitors were generated that act at subnanomolar concentrations with 5000-fold selectivity for p38 over Raf-1 and JNK2 [33]. Thus, the combination of information derived from structural studies and sequence databases has significantly facilitated the development of selective p38 inhibitors.

The unphosphorylated (low-activity) form of p38 binds ATP poorly. Indeed, ATP competes with a tritiated pyridinyl imidazole inhibitor, SB202190, for binding to the active, phosphorylated p38 MAPK, but does not compete with labeled inhibitor binding to the low-activity, unphosphorylated enzyme [34]. Furthermore, β,γ-methyleneadenosine 5′-triphosphate (AMP-PCP) blocks labeling of the active site lysine with fluorosulfonyl benzoyl adenosine (FSBA) in the active form of p38, but fails to prevent FSBA labeling of this lysine in the unphosphorylated enzyme [34]. By contrast, tritiated SB202190 has a similar binding affinity to the low- (K_d = 37 nM) and high- (K_d = 38 nM) activity forms of p38 [34]. Thus, these findings are consistent with poor nucleotide binding to the low-activity form of the enzyme [35], and provide an explanation for the observation that p38 inhibitors have significant cellular efficacy despite intracellular ATP concentrations in the millimolar range. As a consequence of binding the low-activity form, these inhibitors appear to interfere with the activation of p38. Perhaps kinases whose low-activity forms bind ATP poorly will prove the most tractable therapeutic targets.

MEK inhibitors

The development of MEK1 and MEK2 inhibitors has also progressed, although not as rapidly as the development of p38 inhibitors. These efforts have been impeded by the lack of three-dimensional structures for any members of the MEK family. Structural information is particularly important for this class of kinase inhibitors because two of these inhibitors, PD98059 and U0126 (Fig. 2), do not appear to compete with ATP and thus are likely to have a distinct binding site on MEK [5,36]. Detailed structural information on the binding modes of these compounds would significantly enhance the drug design process. Interestingly, in a comparison of multiple kinase inhibitors, the MEK1 and MEK2 inhibitors appeared to be the most specific kinase inhibitors tested because they inhibited the fewest non-target kinases in a panel of 24 kinases [32]. MEKs are attractive targets for therapeutic intervention because of their unusually restricted substrate specificity in that they phosphorylate and regulate only a very small number, in most cases one or two, downstream MAPKs. Enhanced MEK1 and MEK2 activity was detected in a significant number of primary human tumor cells [37]. Thus, MEK1 and MEK2 inhibitors are being developed as therapeutic agents for the treatment of cancer [38].

The first MEK1 and MEK2 inhibitor, PD98059, was identified in an *in vitro* screen for inhibitors of ERK activation [4,36]. Shortly thereafter a second inhibitor, U0126, was found in a cell-based screen for inhibitors of phorbol ester-induced activational protein 1 (AP-1) activity [5]. Neither of these inhibitors is competitive with ATP. They appear to bind to similar sites on MEK1 because labeled U0126 competes for binding with PD98059 [5]. Although U0126 and PD98059 have been shown to block MEK1 phosphorylation and activation of ERK1 and ERK2

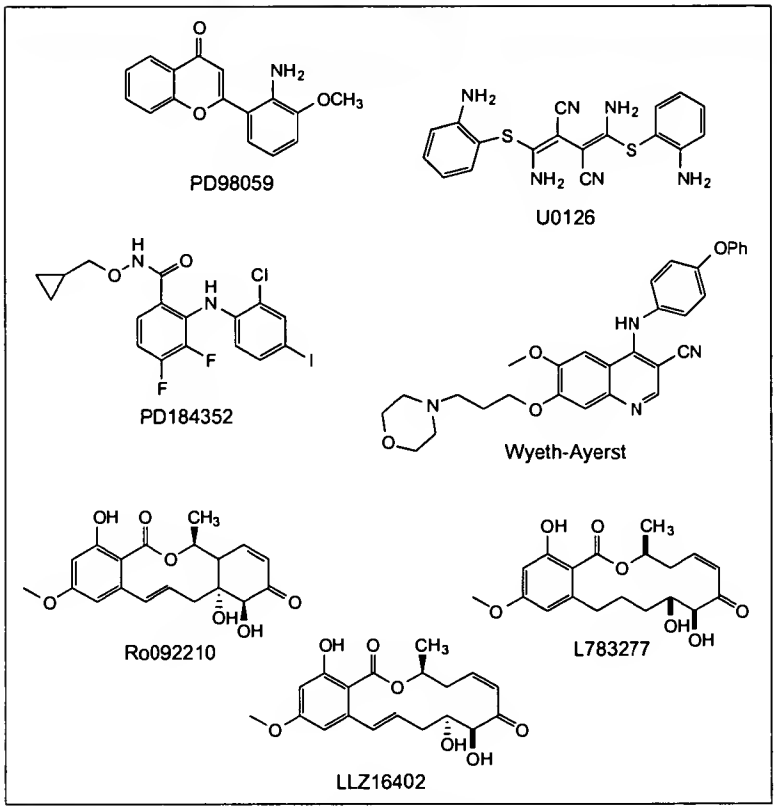

Fig. 2. Mitogan-activated protain kinasa (MAPK)/axtracellular signal-regulatad kinasa (ERK) kinasa (MEK) inhibitors. PD98059, PD184352 and U0126 ara noncompetitiva inhibitors of MEK1 and MEK2. Tha Wyeth-Ayerst compound is reportad to inhibit activa MEK1. Ro092210, LLZ16402 and L783277 era compounds isolatad from microorganisms. Ro092210 and LLZ16402 ara inhibitors of MEK1 and MEK2 that compata with ATP. L783277 has a similar structura to Ro092210 and LLZ16402. L783277 is reported to inhibit Jun-N-tarminal kinasa (JNK)/p38 MAPK pathways upstream of MAPK, but a direct *in vitro* assay of MEK inhibition has not been reported.

both *in vitro* and in *vivo*, there is confusion in the literature regarding the capacity of these inhibitors to prevent activation of MEK1 and MEK2 and their capacity to inhibit active MEK1. Initial reports suggested that PD98059 works by inhibiting phosphorylation of MEK1 by upstream activators and that it could not inhibit the active form of the enzyme [36]. By contrast, U0126 was initially reported to inhibit phospho-MEK1 and constitutively active MEK1 but not phosphorylation of MEK1 by upstream activators [5]. In two more recent studies, U0126 inhibited *in vitro* phosphorylation of MEK1 by upstream activators [32,39]. In these same studies PD98059 did not inhibit recombinant active phospho-MEK1; this could be due, in part, to the limited solubility of PD98059 [32,39]. Neither compound inhibited phosphorylation of MEK1 *in vivo* [39]. Thus, consistent with the data indicating a similar binding site, these compounds appear to inactivate MEK1 through similar allosteric mechanisms. They inhibit catalysis of phosphoryl transfer to ERK1 and ERK2 but *in vivo* might not inhibit phosphorylation of MEK1 and MEK2, and might even enhance phosphorylation of MEK1 and MEK2 [39].

These first-generation MEK inhibitors have been used extensively to attribute biological activities to ERK1 and ERK2 *in vivo* [1,2]. However, both of these inhibitors have recently been shown to inhibit activation of the ERK5 pathway through direct effects on MEK5 [40–42]. In cell-based assays these inhibitors are often used at 5–50 μM, and effects on ERK5 can be detected in this range. In addition, PD98059 inhibits cyclooxygenase 2 (COX-2), further confounding the interpretation of the inhibitory actions of PD98059 [43].

A second-generation MEK1 and MEK2 inhibitor, PD184352 (Fig. 2), has an IC_{50} value below 20 nM, enhanced bioavailability, and also appears to work via an allosteric mechanism [32,38,44]. Elevated ERK1 and ERK2 activity in colon 26 carcinoma cells was inhibited by PD184352 with an IC_{50} of 120 nM, and it inhibited growth of tumors derived from these cells when given orally to mice [44]. Although no inhibition of JNK/SAPK, p38 or Akt was detected, epidermal growth factor (EGF) activation of the MEK5–ERK5 pathway was inhibited at micromolar concentrations [42]. *In vivo* MEK5 phosphorylation also appeared to be enhanced by PD184352 [42]. PD184352 is currently being evaluated in Phase I clinical oncology trials [45].

Wyeth-Ayerst has also identified a series of novel MEK inhibitors that are 3-cyano-4-(phenoxyanilino)quinolines (Fig. 2) [46]. The most potent of these possessed an IC_{50} value of 7 nM with 100 μM ATP in the assay, and an IC_{50} value of 190 nM for inhibition of cell growth. Active MEK1 was reported to be inhibited by this compound [46]. It was not reported whether this compound was competitive with ATP.

MEK inhibitors from microbial extracts

A novel family of MEK inhibitors containing four rings and an essential ketone was identified by two groups in fermented extracts from two different sources (Fig. 2) [47,48]. The inhibitor identified by Roche, Ro092210, has an IC_{50} value below 60 nM with 100 μM ATP in the *in vitro* assay. It is effective at similar or lower concentrations in cell-based assays, including blockade of anti-CD3-induced T-cell activation, the assay used to identify the compound [47]. The inhibitor identified by Merck, L783277, has an IC_{50} of 80 nM, measured with the same ATP concentration as the Roche compound *in vitro*, is slowly irreversible, and inhibits the growth of several epithelial cell lines [48]. Unlike the first MEK1 and MEK2 inhibitors discovered, these compounds are competitive with ATP and also block MEKs 4, 6 and 7 at 4–10-fold the MEK1 and MEK2 IC_{50} value. An antibiotic, LLZ16402, with a very similar structure to both Ro092210 and L783277 (Fig. 2) might also be a MEK inhibitor, although direct *in vitro* inhibition of a MEK by LLZ16402 has not yet been reported. LLZ16402 was identified as an inhibitor of phorbol-ester-induced AP-1 transcription [49].

At concentrations of 25–100 ng ml^{-1}, LLZ16402 inhibited JNK and p38 activation by anisomycin but did not inhibit ERK1 and ERK2 activation by EGF [49].

Thus, these resorcylic acid lactones appear to be produced in multiple microorganisms and might inhibit multiple MEK family members, in some cases with nanomolar potencies. No comprehensive evaluation against multiple MEKs *in vitro* has been reported for these compounds. Thus, their relative selectivity among MEK family members remains unclear.

JNK/SAPK pathway inhibitors

The JNK/SAPK MAPK pathway is also being targeted for small-molecule drug development. SP600125 (Fig. 3), has been reported to inhibit JNK2 at 100 nM *in vitro* and does not inhibit ERK, p38β or IκB kinase (IKK) at micromolar concentrations [50]. Although the cellular IC_{50} value was micromolar, the *in vitro* data suggest that the JNK family might be amenable to small-molecule drug discovery. The development of specific inhibitors for each family of MAPKs and inhibitors that act at different levels in these pathways would greatly facilitate our understanding of the complex interactions of these signaling cascades.

Another small-molecule inhibitor of the JNK pathway, CEP1347 (KT7515) (Fig. 3), has been described [51]. CEP1347 was recently reported to inhibit members of the mixed lineage kinase (MLK) family with IC_{50} values of 23–51 nM for MLK1, 2 and 3 [52]. MLK1, 2 and 3 are upstream activators of the JNK pathway. Thus, this is an initial attempt to target the JNK pathway via inhibition of select MEKKs upstream of JNKs. Because of the large number and diversity of the genes encoding MEKKs it has been difficult to determine their precise *in vivo* roles. Therefore, it is currently unclear which of these enzymes might provide the best therapeutic targets. An exception is Raf-1 for which there is considerable evidence defining its role as a major effector of Ras, the most commonly mutated oncogene in human tumors. Thus, Raf-1 is an attractive target for therapeutic intervention in cancer.

Raf-1 inhibitors

A three-kinase-coupled assay was used by Glaxo Wellcome to identify potent Raf-1 inhibitors in a series of oxindoles (Fig. 3) [53]. Phenol substituents increased inhibitor potency. Several compounds with acidic pK_a values were found to possess IC_{50} values in the low nanomolar range and blocked ERK1 and ERK2 activation in cells in the low micromolar range. A Raf-1 inhibitor that competes with ATP has also been reported by Merck and has an IC_{50} value of 2 nM against recombinant Raf and 0.3–2.0 μM IC_{50} values for anchorage-independent growth [54]. The most advanced Raf-1 inhibitors have been developed in collaboration between Bayer and Onyx. Bay439006 has demonstrated efficacy in cell and animal assays. Bay439006 is currently in Phase I trials in locally advanced or metastatic cancer [55].

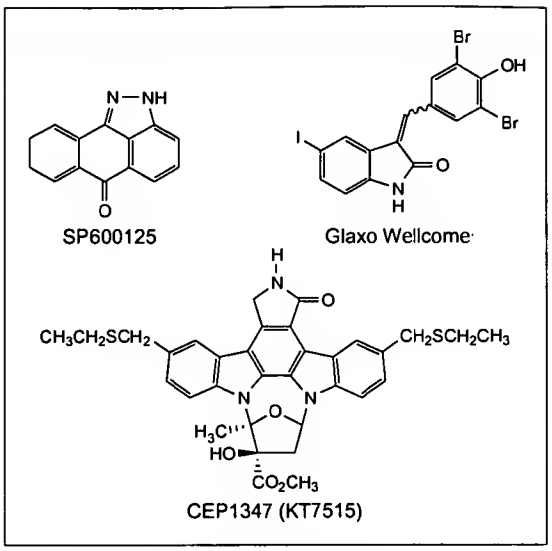

Fig. 3. Othar publishad inhibitors of mitogan-activated protein kinasa (MAPK) pathways. SP600125 was developed by Signal Resaarch Division of Celgena and inhibits Jun-N-terminal kinase 2 (JNK2) *in vitro* with an IC_{50} valua of 100 nM. Tha Glaxo Wallcoma compound inhibits Raf *in vitro* with an IC_{50} value of 9 nM. CEP1347 (KT7515) is an inhibitor of mixad linaaga kinasas 1, 2 and 3 (MLK1, 2 and 3) and is synthasizad by darivation of an indolocarbazola isolated from a natural product produced by tha *Narcodiopsis* bacterium.

Concluding remarks

Less than a decade ago the kinases constituting mammalian MAPK pathways were identified through intense efforts to understand the molecular events underlying cellular responses to extracellular signals. During this decade the kinases constituting MAPK pathways have come to be appreciated as key cellular signal transducers and thus attractive targets for drug development. Successful drug development has required the demonstration that the difficulties presented by a large gene family with a highly conserved catalytic core could successfully be targeted with specific and potent small-molecule inhibitors. The elucidation of both multiple kinase structures and the sequencing of the human genome have aided and will continue to facilitate this process considerably. These efforts are now beginning to bear fruit with the initiation of clinical trials in multiple human diseases. It is currently unclear whether targeting a single signal transduction pathway will be efficacious in many disease states. Many of these emerging drugs will probably be used in combination therapy, particularly in oncology. The determination of the clinical protocols in combination therapy and the relevance of preclinical basic research to these clinical protocols is still relatively unclear. Nevertheless, the outcome of clinical trials of compounds inhibiting MAPK pathways is of significant interest to both the basic and the clinical scientific communities. Their positive outcome would be a triumph of translating basic scientific understanding of cellular function into successful human therapies.

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Chemical names

SB202190: phenol, 4-[4-(4-fluorophenyl)-5-(4-pyridinyl)-1*H*-imidezol-2-yl]
SB203580: pyridine, 4-[4-(4-fluorophenyl)-2-[4-(methylsulfinyl)phenyl]-1*H*-imidazol-5-yl]
SB216995: pyridine, 4-[1-(cyclopropylmethyl)-4-(4-fluorophenyl)-1*H*-imidezol-5-yl]
SB218655: 2-pyrimidinamine, 4-[1-(cyclopropylmethyl)-4-(4-fluorophenyl)-1*H*-imidazol-5-yl]
SB220025: 2-pyrimidinemine, 4-[4-(4-fluorophenyl)-1-(4-piperidinyl)-1*H*-imidazol-5-yl]
VK19911: pyridine, 4-[4-(4-fluorophenyl)-1-(4-piperidinyl)-1*H*-imidezol-5-yl]